Micellar Complexes of Human Apolipoprotein A-I with Phosphatidylcholines and Cholesterol Prepared from Cholate-Lipid Dispersions

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Micellar complexes of human apolipoprotein A-I and phosphatidylcholine, with or without cholesterol, were prepared by adding apolipoprotein A-I (apo A-I) to sodium cholate-lipid mixtures. Cholate was removed by dialysis and the apo A-I-lipid complexes were isolated by gel filtration chromatography or by density gradient ultracentrifugation. The lipid mixtures consisted of dipalmitoylphosphatidylcholine or egg yolk phosphatidylcholine in the presence of various molar ratios of cholesterol. The formation of complexes was examined at different phosphatidylcholine (PC)-to-apo A-I ratios, PC-to-cholate ratios, and cholate concentrations.

Yields of complexes were maximal when incubation and dialysis were performed near the transition temperature of the PC.

Upon lipid binding and complex formation, apo A-I experienced a significant increase in α-helix content, and a blue shift in the intrinsic tryptophan fluorescence. In all lipid-protein incubation mixtures, from 80:1 to 75:1, PC/apo A-I (molar ratios), relatively small, stable complexes were present which gave maximum yields at incubation ratios similar to their isolated stoichiometries of 75:1 to 140:1, PC/apo A-I (molar ratios). For the isolated complexes, molecular weights were determined by sedimentation equilibrium to be in the range from 220,000 to 260,000; fluorescence polarization using the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene showed a broadened and shifted gel to liquid-crystalline phase transition, characteristic of micellar complexes of apo A-I with PC. Complexes prepared using apo A-I, covalently labeled with 5-di-methylaminonaphthalene-1-sulfonyl chloride, had an overall particle rotational relaxation time of 530 ns. On electron micrographs, the complexes, negatively stained with phosphotungstate, appeared as lamellar, discoidal particles.

The interest in apolipoprotein A-I interactions with lipids is due to the assumption that this major protein of human and animal high density plasma lipoproteins probably defines the structure and function of the high density lipoprotein class. The surfactant-like action of apo A-I on multilamellar liposomes or unilamellar vesicles of DMPC to produce micellar, high density particles has been well documented (1–5).

Recent work has indicated that the nature and size of the apo A-I-DMPC complexes depend on the PC-to-apolipoprotein ratio in the reaction mixture (5–7) and on the properties of the lipid, including the size of the lipid particles (7) and their physical state (8, 9). In addition to DMPC, shorter chain, saturated PCs form micellar products with apo A-I (10, 11) quite readily; on the other hand, DPPC has to be at its transition temperature and in unilamellar vesicle form to give good yields of micellar products (8); palmitoyloleoylphosphatidylcholine does not spontaneously react with apo A-I to give micellar particles even near its transition temperature (12); and egg-PC gives very poor yields of small particles when sonicated with apo A-I at all accessible temperatures. Incorporation of cholesterol into apo A-I-PC micellar complexes has also been described by several laboratories (13–16), but it is evident that beyond 20–30 mol % cholesterol contents the reaction decreases markedly even with DMPC.

The purpose of this work was to develop a general method for the preparation, in good yields, of micellar, stable complexes between various PCs containing up to 20 mol % cholesterol and apo A-I. These complexes are used in the following paper as models of discoidal, nascent HDL substrates for the plasma enzyme lecithin cholesterol acyltransferase.

EXPERIMENTAL PROCEDURES

RESULTS

Fig. 1 (A through C) shows the elution profiles on a Sepharose CL-4B column of DPPC-apo A-I mixtures after cholate removal. For decreasing initial molar ratios of DPPC/A-I (300:1 to 130:1), it is apparent that each mixture includes a lipoprotein complex eluting at fraction 44 ± 2 and having an average DPPC/apo A-I molar ratio of 110 (± 20):1. The elution profiles also indicate the presence of complexes containing higher, variable DPPC/A-I ratios, eluting in fractions 34 to 42. Fig. 2 (A through E) shows the isopycnic density...
gradient profiles of the same DPPC-apo A-I mixtures, including molar ratios of 600:1 and 75:1, DPPC/apo A-I. These results indicate, in agreement with the gel filtration data, that stable complexes with average DPPC/apo A-I molar ratios of 120 (± 20):1 can be isolated at density 1.085 ± 0.010 g/ml from all the reaction mixtures. It is apparent that the yields of these complexes are maximal at initial DPPC/apo A-I ratio equal to or just greater than the stoichiometries of the isolated complex. At higher incubation ratios of DPPC/apo A-I, there are complexes of lower density and higher molar ratios (lipid to protein) formed as well (Fig. 2, A–C). For the initial molar ratio of 75:1 DPPC/apo A-I, free apo A-I sediments to the bottom of the centrifuge tubes (Fig. 2, E).

Routinely, approximately 80% of the initial apo A-I was recovered in the smaller complex peak at an initial DPPC/apo A-I ratio of 135:1. These complexes were pooled over 5 column fractions and were termed “micellar,” because of their similarity to DMPC-apo A-I complexes (2, 6, 7); they were used exclusively in the physical characterization studies.

The isolation, by density gradient centrifugation, of complexes which included different mole per cent cholesterol in the incubation mixtures is illustrated in Fig. 3. Cholesterol mole per cent throughout this paper gives the number of cholesterol molecules/100 lipid molecules (PC + cholesterol). In the tables, the cholesterol content, for the sake of simplicity of presentation, is given as moles/mol of apo A-I.

Egg-PC gives very similar results to the DPPC systems. Complexes of egg-PC-apo A-I elute at fraction 56 on the Bio-Gel A-5m column (equivalent to fraction 45 on the Sepharose CL-4B column), have a buoyant density of 1.090 ± 0.010 g/ml, and a PC-to-apo A-I molar ratio of 55 (± 15):1. Complex yields decreased with increasing cholesterol contents, although better yields were obtained for the egg-PC-cholesterol than for the DPPC-cholesterol mixtures at 20 mol % cholesterol. For the micellar complexes, the gel filtration and density gradient profiles had relatively constant PC/apo A-I as well as PC/cholesterol ratios across the peaks.

We also determined that similar complexes of egg-PC-apo A-I form over a range of cholate concentrations from 4:1 to 1:2, PC/cholate (molar ratios). All the above results on incubation conditions and micellar complex compositions and yields are summarized in Table I.

Structural changes in apo A-I upon complex formation were followed by intrinsic fluorescence and circular dichroism measurements on the free protein and on the isolated complexes. As shown in Table II, there was a 5 nm blue shift of the maximum fluorescence wavelength of apo A-I going from the free (333 nm) to the lipid-bound state (328 nm). Percentage of α-helix content increased concomitantly from 37 to 75%. Circular dichroism measurements on unfractionated mixtures of DPPC and apo A-I, after cholate removal, indicated increasing molar ellipticities with increasing molar ratios of PC/apo A-I (data not shown). Maximum ellipticity was reached at 115 (± 10):1, DPPC/apo A-I (molar ratios). This saturation ratio corresponds to the stoichiometry of the micellar complexes isolated by gel filtration and density gradient centrifugation.

At lower DPPC/apo A-I ratios, free protein contributes to the observed molar ellipticity values, whereas at higher molar ratios all the protein is found in complexes with lipid.

Lipid phase changes in DPPC-apo A-I micellar complexes, with and without cholesterol, were observed by means of the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene as a function of temperature (see Fig. 4). Relative to the phase behavior of DPPC multilamellar liposomes, which have a \( T_m \) at around 41 °C, the DPPC-apo A-I complexes have a broadened transition of lower amplitude, and with a midpoint shifted toward a higher temperature (44 °C). In the case of the cholesterol-containing complexes, the phase transition is still perceptible, but the broadening and decrease in amplitude are even more marked than for DPPC-apo A-I. The midpoints of the linear regions of the plots, shown in Fig. 4, were taken to be the midpoints of the transitions.

Rotational relaxation times \( (\rho_e) \) were determined for apo A-I-Dns and for its complexes with egg-PC and with egg-PC plus cholesterol from fluorescence polarization measurements as a function of temperature/viscosity ratios (varied isothermally), and from lifetime values, using the Perrin expression (21, 30). Rotational relaxation times for free apo A-I-Dns and its complexes with lipid were 52 ± 5 and 530 ± 60 ns, respectively. These values correspond to 27 and 58 Å Stokes radii, as indicated in Table III.

The ultrastructure of the micellar complexes was examined by negative staining and transmission electron microscopy (Fig. 5). The electron micrographs show the characteristic discoidal particles, arranged in stacks which have already been demonstrated for DMPC-apo A-I complexes (3, 6) and

![Fig. 5. Electron micrographs of isolated micellar complexes of DPPC-apo A-I (left) and egg-PC-apo A-I (right) at a magnification of 240,000-fold.](http://www.jbc.org/)
for discoidal or "nascent" HDL preparations (31, 32).

Molecular weights, determined for isolated micellar complexes by sedimentation equilibrium ultracentrifugation, are given in Table II. The plots of $\ln A_{200}$ versus $r^2$ were nearly linear for the PC-apo A-I complexes, but exhibited some curvature for the cholesterol-containing complex, suggesting some size heterogeneity in the sample.

**DISCUSSION**

We have demonstrated that micellar lipid-apo A-I complexes can be prepared in good yields from apo A-I and lipid dispersions with sodium cholate. Under simple incubation conditions, the lipids used in our studies, DPPC and egg-PC plus cholesterol, do not react readily with apo A-I to form small, micellar products.

The average molar ratio of PC to apo A-I in complexes depends on the initial lipid-to-protein ratio; however, a stable, minimum size complex is present in all the reaction mixtures. The yields of these micellar complexes are highest at incubation molar ratios (PC/apo A-I) near the stoichiometry of the isolated complexes, at cholesterol contents of less than 20 mol % (see Table I), and at incubation temperatures approaching the transition temperature of the corresponding PC. Whether incubation molar ratios above 200:1, PC/apo A-I produce larger micellar complexes and/or vesicular complexes has not been determined in this work.

The physical properties and morphology of the isolated micellar products, summarized in Tables II and III and shown in Figs. 4 and 5, are very similar to those of discoidal complexes of DMPC-apo A-I, characterized by our laboratory (2, 6, 13) and by other investigators (1, 3–5). Here we find that egg-PC, which contains longer chain and unsaturated fatty acids, forms complexes of somewhat lower PC/apo I molar ratios and of slightly larger average size than DPPC. Taking into account the molecular weights and average stoichiometries of the complexes, we calculate that DPPC-apo A-I complexes contain 2 apo A-I molecules/complex, whereas the egg-PC-apo A-I complexes contain on the average 2.5 apo A-I's. Since the errors in the molecular weight and composition determination are quite high (of the order of ±15% each), the calculated 2.5 apo A-I average content of apo A-I per particle may not necessarily represent a mixed population of complexes, some containing 2 and some 3 apo A-I's. Nevertheless, the irregular sizes of discs observed by electron microscopy (Fig. 5) for the egg-PC-apo A-I sample suggest that there is some heterogeneity in this preparation. The presence of 20 mol % cholesterol in egg-PC-apo A-I complexes does not change significantly the lipid/apo A-I weight ratio, but gives a more heterogeneous distribution of particles as indicated by electron micrographs (not shown) and by the curvature of the $\ln A_{200}$ versus $r^2$ plots used in the molecular weight determinations.

The mechanism by which apo A-I interacts with lipid-cholate mixtures and eventually forms the micellar products is not known, but it probably has to do with a facilitated penetration of apo A-I between the PC acyl chains. It is well known that apo A-I interacts optimally with DMPC (4, 8, 12) and DMPC-DPPC mixtures (8, 12) at the transition temperature of the lipids, i.e. where gel and liquid-crystalline phases co-exist and where lattice defects allow the penetration of the apolipoprotein into the bilayer (4). Cholate disrupts the PC lattice by intercalation and/or solubilization. The egg-PC-cholate mixtures used in this work can reportedly exist in three different states: below 1.2, PC/cholate, small spherical micelles predominate (33); from 1.2 to 2.1 PC/cholate, bilayer discs of PC plus cholate, stabilized by an annular arrangement of cholate molecules are formed (34, 35); and at ratios above 2:1, PC/cholate, PC multilamellar structures with intercalated cholate are present (34, 35) (all molar ratios). The fact that all these PC-cholate states eventually form similar complexes with apo A-I indicates that, whatever the pathway of the interaction, the final, most thermodynamically stable products are the discoidal, micellar complexes.

The present method can be easily adapted to the preparation of synthetic complexes containing various PCs, apolipoproteins, and cholesterol levels, which will find numerous applications as enzyme substrates and as models of nascent HDL. We describe in the following paper (36) the reaction of PC-cholesterol-apo A-I complexes with lecithin cholesterol acytransferase. We show that these complexes are excellent substrates for the enzyme which will permit a close examination of the mechanism of cholesterol ester formation in plasma. The use of these small, soluble, lipid-containing complexes may be extended to other enzymes as well. As models of nascent HDL, these complexes can be subjected to enzymatic activity (36) and to lipid and protein transfers, under controlled conditions, to explore the transformations of nascent HDL, and perhaps the origin of HDL subclasses in circulation.

**REFERENCES**

Micellar Complexes of Apo A-I with Lipids

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SPELLENDON

MICELLAR COMPLEXES OF HUMAN APOLIPOPROTEIN A-I WITH PHOSPHATIDYLCHOLINE AND CHOLESTEROL PREPARED FROM CHOLESTEROL-LIPID DISPERSIONS

Chen, L. and Apo A-I Complexes

APPARATUS Preparations — The procedure for the purification of bovine apo A-I from pooled serum was similar to that described by Reynolds et al. (117); the precipitation was achieved by dialysis of apo A-I against 0.1 M sodium sulfate followed by a second dialysis against 0.2 M sodium carbonate. The precipitate was then dissolved in 0.5 M phosphate buffer, pH 7.4. The second step in the purification was a fractionation of the apo A-I protein by centrifugation at 50,000 g and 370,000 g, using either sodium chloride or potassium chloride gradients. The fractionation of apo A-I was performed from the micellar phase of 370,000 g, and the final complex was purified by the method of Chen et al. (22).

Lipid-Cholesterol Apo Complexes — The micellar complexes were prepared by adding an aliquot of a known concentration of apo A-I containing 1 mol % apo A-I to a known concentration of a phospholipid and cholesterol mixture containing 25 mol % each. After the addition, the mixture was allowed to react for 1 hour at 37°C. The final complex was prepared by dialysis against 0.1 M sodium carbonate and 0.2 M sodium carbonate at 4°C. The final complex was then dialyzed against 0.1 M sodium carbonate and 0.2 M sodium carbonate at 4°C.

Physical Properties of Apo A-I Complexes

Physical Properties of Apo A-I Complexes with Lipids

Table I

<table>
<thead>
<tr>
<th>Component</th>
<th>PC/mg apo A-I</th>
<th>Cholesterol/mg apo A-I</th>
<th>Total Lipid/mg apo A-I</th>
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<tr>
<td>Apo A-I</td>
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<tr>
<td>PC/mg apo A-I</td>
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<tr>
<td>Total Lipid/mg apo A-I</td>
<td>900</td>
<td>1800</td>
<td>2700</td>
</tr>
</tbody>
</table>

a: Calculated from data in Table III.

b: Calculated from data in Table II.

c: Calculated from data in Table I.

**Notes:**
- The data in Table I represent an average of at least three determinations.
- The data in Table II were calculated from data in Table I.
- The data in Table III were calculated from data in Table II.

Supplementary Table

<table>
<thead>
<tr>
<th>Component</th>
<th>PC/mg apo A-I</th>
<th>Cholesterol/mg apo A-I</th>
<th>Total Lipid/mg apo A-I</th>
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<tr>
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<td>Total Lipid/mg apo A-I</td>
<td>900</td>
<td>1800</td>
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</tr>
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</table>

**Notes:**
- The data in Table III represent an average of at least three determinations.
- The data in Table III were calculated from data in Table II.
- The data in Table III were calculated from data in Table I.

**References:**
- Reynolds, J. et al. (117)
- Chen, L. et al. (22)

**Acknowledgments:**
- The authors gratefully acknowledge the technical assistance of Mr. J. Reynolds and Mr. L. Chen in the preparation of the micellar complexes.

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Micellar Complexes of Apo A-I with Lipids

Figure 1—Fractionation profiles from a Sepharose 6B column (1.6 x 35 cm) of DPPC reaction mixture with apo A-I, after dialysis. Results of 25, 50, and 70% recoveries to the column excluded volume, retentate volume of small unilamellar liposomes, and retentate volume of free apo A-I, respectively. The distribution volume ratios of DPPC:apo A-I were 300:1, 600:1, and 1200:1 in the panels A, B, and C, respectively, across the elution peaks in terms of DPPC:apo A-I (MM) (△). Fractions 42-46 were pooled for subsequent experiments.

Figure 2—Fractionation of DPPC:apo A-I reaction mixtures by density gradient centrifugation in a linear dextran gradient. The reaction mixture ratios of DPPC:apo A-I were: 300:1 (panel A), 600:1 (panel B), 1200:1 (panel C), 2400:1 (panel D), and 4800:1 (panel E). The compositions of the fractions are shown across the peaks in terms of DPPC:apo A-I (MM) (△). Fractions 42-46, including the 0-1.095 g/ml fraction, were pooled for subsequent experiments.

Figure 3—Fractionation of DPPC:cholesterol:apo A-I reaction mixtures by density gradient centrifugation in a linear dextran gradient. The reaction mixture ratios of DPPC:cholesterol:apo A-I were: 135.8:1:1 (panel A), 135.8:1:2 (panel B), and 135.8:1:3 (panel C). The compositions of the fractions are shown across the peaks in terms of cholesterol:apo A-I (MM) (△). Fractions 42-46 were pooled for subsequent experiments.

Figure 4—Phase transition behavior of multilamellar DPPC liposomes. □, isolated multilamellar liposomes; ●, isolated multilamellar liposomes containing 20 mol% cholesterol; △, isolated multilamellar liposomes containing 20 mol% cholesterol and 3 mol% apo A-I. The fluorescence polarization ratio of apo A-I fluorophore was determined by the sample in a ratio of 390:1. The percentage of apo A-I, relative to 0%, was measured as a function of temperature.
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